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CD80 and CD86 costimulatory molecules regulate crescentic glomerulonephritis by different mechanisms

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CD80 and CD86 costimulatory molecules regulate crescentic glomerulonephritis by different mechanisms.

Background. CD80 and CD86 costimulatory molecules have been shown to affect the induction of Th1-mediated crescentic antiglomerular basement membrane (GBM) antibody-initiated glomerulonephritis (GN). The aim of the current studies was to define the mechanisms by which CD80 and CD86 regulate the development of this disease.

Methods. Anti-GBM GN was induced in CD80−/−, CD86−/−, and CD80/86−/− mice, as well as in C57BL/6 controls. Renal injury and immune responses were assessed after 21 days. To examine whether costimulation by OX40-ligand compensates for the absence of CD80 and CD86 in inducing GN, OX40-ligand was blocked in wild-type and CD80/86−/− mice.

Results. Crescentic GN and glomerular accumulation of CD4+ T cells and macrophages were attenuated in CD80−/− mice, correlating with significantly enhanced apoptosis and decreased proliferation of spleen CD4+ T cells. GN was exacerbated in CD86−/− mice, which was associated with attenuated IL-4 and enhanced IFN-γ levels. In contrast, CD80/86−/− mice developed crescentic GN similar to that in controls. Inhibition of OX40-ligand exacerbated GN in wild-type mice by enhancing IFN-γ production, and attenuated disease in CD80/86−/− mice by reducing glomerular CD4+ T-cell and macrophage accumulation.

Conclusion. CD80 is pathogenic in crescentic GN by enhancing survival and proliferation of CD4+ T cells, whereas CD86 is protective by enhancing Th2 and attenuating Th1 responses. Furthermore, in the presence of CD80 and CD86, OX40-ligand attenuates, whereas in their absence it enhances GN, suggesting that, in the absence of CD80 and CD86, the OX40/OX40-ligand pathway is an alternative costimulatory pathway in inducing crescentic GN.

Glomerulonephritis (GN) is a major cause of end-stage renal failure. Immune responses to self or foreign antigens (Ag) in glomeruli result in many different forms of GN, with the most severe and rapidly progressive type being crescentic nephritis. While some valuable insight into the pathogenesis of crescentic GN has been provided by observations of human immunopathology, much of our insight into the mechanisms of crescentic nephritis comes from experimental models. The most widely used animal model of crescentic GN is antiglomerular basement membrane (GBM) antibody (Ab)-initiated GN, where heterologous anti-GBM Ab acts as a planted Ag in glomeruli. In C57BL/6 mice, the development of crescentic anti-GBM GN is directed by CD4+ Th1 cells, attenuated by Th2 cytokines, and is strongly dependent on glomerular infiltration of delayed type hypersensitivity (DTH) effectors, CD4+ T cells, and macrophages, while it is independent of autologous Ab deposited along the GBM [1–4].

Optimal activation of naïve CD4+ T cells requires engagement of the T-cell receptor, as well as nonantigen-specific costimulatory signals. The best-characterized costimulatory signal is provided by CD80 (B7-1) and CD86 (B7-2) expressed on antigen-presenting cells. Interaction between these ligands and T-cell receptors, CD28 and CTLA-4, either enhances or down-regulates T-cell responses, respectively [5–7]. CD80 and CD86 can affect disease outcome by promoting T-cell survival and proliferation, and by altering the Th1/Th2 balance. For example, disruption of CD28/B7 costimulation inhibited proliferation of Ag-specific T cells and, consequently, reduced the severity of experimental autoimmune encephalomyelitis (EAE) [8]. In addition, the absence of CD80/86 costimulation exacerbated autoimmune diabetes due to reduced IL-4 and enhanced IFN-γ production [9], while it attenuated EAE due to the inhibition of Th1 and up-regulation of Th2 cytokines [10]. Studies investigating the individual roles of CD80 and CD86 have shown that these molecules can have distinct or overlapping functions during an adaptive immune response depending on the disease model studied and the immune mechanisms involved. For example, blockade of CD80 or CD86 attenuated or enhanced the development of EAE,

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respectively [11], while combined blockade of both CD80 and CD86 attenuated collagen-induced arthritis [12]. Furthermore, it has been shown that CD80/86 costimulation is not always required for the development of T-cell–mediated diseases, suggesting the existence of alternative costimulatory pathways, such as the OX40/OX40-ligand pathway, which has been demonstrated to be of particular importance in T-cell responses and to synergize with CD28 costimulation, but at the same time to provide a CD28-independent signal [13–17].

The role of CD80 and CD86 costimulation has also been investigated in several models of GN, including experimental autoimmune GN (EAG) [18, 19], lupus nephritis [20–24], renal transplantation [25], and anti-GBM globulin GN [26]. In the anti-GBM GN model, inhibition of both CD80 and CD86 had no effect on disease development, while anti-CD80 mAb treatment attenuated, and anti-CD86 treatment exacerbated, renal injury [26]. These results, however, could not be explained by changes in humoral or cell-mediated systemic immunity. The current studies aimed to define the mechanisms by which CD80 and CD86 regulate crescentic anti-GBM GN by using mice genetically deficient in either CD80 or CD86, as well as both CD80 and CD86, and to investigate the role of an alternative costimulatory molecule, OX40-ligand (OX40-L), in the absence of CD80 and CD86. They demonstrate that CD80 has a pathogenic role in crescentic GN by promoting survival and proliferation of CD4+ T cells, while CD86 has a protective role by promoting Th2 and attenuating Th1 responses. They also show that crescentic GN can develop independently of CD80 and CD86, and that the OX40/OX40-L pathway is an alternative costimulatory pathway for mediating immune responses that induce crescentic GN in the absence of both CD80 and CD86.

METHODS

Experimental design

Eight- to 10-week-old male mice were used for experiments. Mice deficient in CD80 (CD80−/−), CD86 (CD86−/−), and CD80 and CD86 (CD80/86−/−) on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type (wt) C57BL/6 mice were obtained from Monash University Animal Services (Melbourne, Australia).

Anti-GBM GN was initiated in mice by an IV injection of 14 mg of sheep antimouse GBM globulin into the tail vein. Renal injury and immune responses were assessed 21 days later. GN was induced in CD80−/− ($N = 11$), CD86−/− ($N = 8$), CD80/86−/− ($N = 12$), and C57BL/6 control mice ($N = 7$) to investigate the role of CD80 and CD86 during the development of crescentic GN. Nonimmunized C57BL/6 mice ($N = 8$) provided normal controls without GN. To determine whether the OX40/OX40-L pathway provides a compensatory signal in the absence of CD80 and CD86 costimulation, OX40-L was blocked in wt and CD80/86−/− mice developing anti-GBM GN. Each mouse received intraperitoneally (IP) 250 μg of protein G purified inhibitory rat antimouse OX40-L mAb [RM134L; provided by Dr. William Heath, Walter and Eliza Hall Institute of Medical Research (WEHI), Melbourne, Australia], which had been previously characterized [13] or control Ab (protein G purified rat IgG). Treatment was started on the day of anti-GBM globulin administration and continued every second day until the end of the experiment. The following groups of mice were studied: (*1*) normal nonimmunized C57BL/6 mice without GN ($N = 8$); (*2*) wt control mice with GN receiving rat IgG ($N = 7$); (*3*) CD80/86−/− control mice with GN receiving rat IgG ($N = 9$); (*4*) wt mice with GN receiving anti-OX40-L mAb ($N = 6$); (*5*) CD80/86−/− mice with GN receiving anti-OX40-L mAb ($N = 8$).

Assessment of renal injury

Glomerular crescent formation was assessed on periodic acid–Schiff's (PAS) reagent-stained, Bouin's fixed 3-μm thick paraffin sections. Glomeruli were considered to exhibit crescent formation if 2 or more cell layers were observed in Bowman's space. For each animal, a minimum of 50 glomeruli was counted to determine the percentage of crescentic glomeruli. Proteinuria (mg protein/24 h) was measured by a modified Bradford's method on urine collected during the final 24 hours of experiments. Serum creatinine concentrations (μmol/L) were measured by an enzymatic creatininase assay.

Glomerular accumulation of macrophages and CD4+ T cells

Macrophages and CD4+ T cells were demonstrated in glomeruli by 3-layer immunoperoxidase staining of periodate-lysine paraformaldehyde (PLP)-fixed frozen 6-μm thick kidney sections, as previously described [2, 27]. M1/70 mAb (rat IgG2b antimouse Mac-1; ATCC, Manassas, VA, USA) was used for macrophages, whereas GK1.5 mAb (rat IgG2b antimouse CD4; ATCC) was used to detect CD4+ T cells. Isotype-matched control Ig (rat IgG2b; Pharmingen, San Diego, CA, USA) served as a negative control. A minimum of 20 glomeruli were assessed per animal and results expressed as cells per glomerular cross section (c/gcs).

Assessment of circulating antigen-specific antibody levels

Circulating mouse antisheep globulin Ig levels (dilutions 1:50 to 1:1600) were assessed by enzyme-linked immunosorbent assay (ELISA) using serum collected at the end of experiments, as previously described [28], using horseradish peroxidase (HRP)-conjugated sheep

antimouse Ig (1:2000; Amersham, Little Chalfont, UK). HRP-conjugated goat antimouse IgG1 (1:4000; Southern Biotechnology Assoc., Birmingham, AL, USA) was used to detect IgG1 (1:50 dilution). IgG2a (1:50 dilution) was detected with biotinylated rat antimouse IgG2a (Pharmingen), followed by Extravidin (Sigma, Castle Hill, NSW, Australia), biotinylated mouse anti-avidin Ab (Sigma), and Extravidin-HRP (Sigma). Sera from nonimmunized C57BL/6 mice were tested to provide baseline Ab levels.

Production of IFN-γ and IL-4 by cultured splenocytes

Spleens were removed from mice at the end of experiments and single cell suspensions obtained. Splenocytes (4×10^6 cells/mL/well) were cultured in Dulbecco's modified Eagle's medium (DMEM) (10% fetal calf serum, FCS) in the presence of 10 μg/mL sheep globulin for 72 hours. The concentrations of IFN-γ and IL-4 in splenocyte supernatants were measured by ELISA, as previously described [29], using the following mAbs: rat antimouse IFN-γ (R46A2; Pharmingen), biotinylated rat antimouse IFN-γ (XMG1.2; Pharmingen), rat antimouse IL-4 (11B11; ATCC), and rat antimouse IL-4 (BVD6; DNAX, Palo Alto, CA, USA).

Apoptosis of CD4+ T cells

Apoptosis of CD4+ cells was assessed by Annexin-V staining according to manufacturer's instructions. Briefly, isolated splenocytes were surface stained with PE-conjugated antimouse CD4 Ab (L3T4; Pharmingen), washed, and stained with a solution containing Annexin-V-Flous (Roche Diagnostics, Penzberg, Germany) and propidium iodide (PI). Analysis was performed by flow cytometry. Results are expressed as percentage of Annexin-V+ PI − CD4+ cells.

In vivo proliferation of CD4+ T cells

Mice were injected IP with 1 mg of bromodeoxyuridine (BrdU) (Sigma) in saline 48, 36, 24, and 12 hours before the end of experiments. As a negative control, one mouse was injected with saline only. Isolated splenocytes were surface stained with PE-conjugated antimouse CD4 Ab (Pharmingen) for 1 hour on ice, washed, then resuspened in 50 μL of cold 150 mmol/L NaCl and fixed by dropwise addition of 0.5 mL cold 95% ethanol. They were incubated on ice for 5 minutes, washed, then incubated with phosphate-buffered saline (PBS) containing 1% paraformaldehyde and 0.01% Tween for 30 minutes at room temperature. Cells were pelleted and incubated with 20 μL of fluoroscein isothiocyanate (FITC)-conjugated anti-BrdU mAb/DNase (Becton Dickinson, Franklin Lakes, NJ, USA) for 30 minutes at room temperature in the dark. They were washed twice, resuspended in PBS, and analyzed by flow cytometry. Results are expressed as percentage of BrdU+ CD4+ cells.

Expression of CD80, CD86, and OX40-L

Surface expression of CD80, CD86, and OX40-L on splenocytes from mice injected with anti-GBM globulin was determined by flow cytometry. Spleens were obtained from mice and singe cell suspensions prepared. Cells (1×10^6) were washed and incubated with hamster anti-CD80 (16-10A1), rat anti-CD86 (GL1), or rat anti-OX40-L mAb (RM134L). After washing, cells were incubated with biotinylated rabbit antirat IgG Ab (Dako, Carpinteria, CA, USA) or biotin-conjugated rabbit antihamster IgG (Sigma) diluted in 10% mouse serum, washed, incubated with streptavidin-FITC (Dako), and analyzed by flow cytometry.

Statistical analysis

Results are expressed as the mean ± SEM. One-way analysis of variance (ANOVA), followed by Dunnett's or Bonferroni's multiple comparison test, was used for statistical analysis (GraphPad Prism; GraphPad Software, Inc., San Diego, CA, USA). Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

C57BL/6 mice develop crescentic GN

C57BL/6 control mice developed crescentic GN (Figs. 1C and 2A), with 20.3 ± 2.5% of glomeruli being affected by crescent formation. They had significant proteinuria (4.8 ± 1.0 mg/24hours vs. normal mice without GN, 1.6 ± 0.2 mg/24hours, $P < 0.05$), as well as macrophages (1.9 ± 0.2 c/gcs) and CD4+ T cells (0.23 ± 0.03 c/gcs) present in glomeruli.

CD80−/− mice develop attenuated GN

Development of GN in CD80−/− mice was attenuated when compared to C57BL/6 controls. Glomerular macrophages (1.9 ± 0.2 vs. 1.0 ± 0.16 c/gcs, $P < 0.05$, Fig. 1A), CD4+ T cells (0.23 ± 0.03 vs. 0.10 ± 0.01 c/gcs, $P < 0.01$, Fig. 1B), and crescents (20.3 ± 2.5 vs. 9.6 ± 1.8%, $P < 0.01$, Figs. 1C and 2B) were reduced in the absence of CD80, although no changes were observed in proteinuria (4.8 ± 1.0 vs. 5.8 ± 0.9 mg/24hours) or serum creatinine (21.7 ± 0.9 vs. 22.6 ± 1.8 μmol/L). Compared to C57BL/6 controls, CD80−/− mice had significantly enhanced CD4+ T-cell apoptosis (5.5 ± 0.4 vs. 11.3 ± 0.6%, $P < 0.0001$, Fig. 3A) and decreased proliferation (16.7 ± 0.7 vs. 12.1 ± 1.2%, $P = 0.017$, Fig. 3C) in the spleen. IFN-γ (Fig. 4A) and IL-4 (Fig. 4B) production by cultured splenocytes, as well as circulating Ig, IgG1, and IgG2a

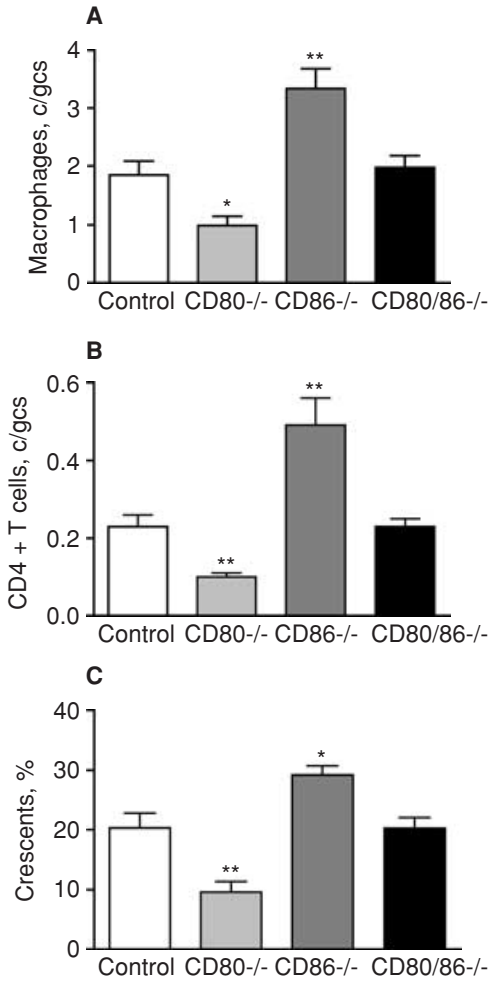

Fig. 1. Glomerular macrophages, CD4+ T cells, and crescents in C57BL/6 mice with GN, and in CD80−/−, CD86−/−, and CD80/86−/− mice. Macrophages (*A*), CD4+ T cells (*B*), and crescents (*C*) were significantly reduced in CD80−/−, markedly increased in CD86−/−, and unaffected in CD80/86−/− mice compared to controls. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

levels (Fig. 5A, Table 1) were not significantly affected in CD80−/− mice.

GN is exacerbated in CD86−/− mice

Glomerular injury was augmented in CD86−/− mice compared to C57BL/6 controls. CD86−/− mice had a trend toward elevated proteinuria (4.8 ± 1.0 vs. 8.3 ± 1.4 mg/24hours, $P = 0.06$) and serum creatinine (21.7 ± 0.9 vs. 28.7 ± 6.4 μmol/L), and developed augmented glomerular infiltration of macrophages (1.9 ± 0.2 vs. 3.3 ± 0.3 c/gcs, $P < 0.01$, Fig. 1A) and CD4+ T cells (0.23 ± 0.03 vs. 0.49 ± 0.07 c/gcs, $P < 0.01$, Fig. 1B), as well as increased crescent formation (20.3 ± 2.5 vs. 29.2 ± 1.5%, $P < 0.05$, Figs. 1C and 2C). Apoptosis of splenic CD4+ cells was reduced in CD86−/− mice compared to C57BL/6 controls (3.2 ± 0.1 vs. 1.7 ± 0.2%, $P < 0.001$, Fig. 3B), as was proliferation (22.7 ± 2.2 vs. 13.9 ± 1.2%, $P < 0.01$, Fig. 3D). CD86−/− mice had markedly enhanced splenocyte IFN-γ production (177 ± 86 vs. 1202 ± 383 pg/mL, $P < 0.01$, Fig. 4A) and a significant decrease in IL-4 (12.4 ± 5.0 vs. 2.0 ± 1.5 pg/mL, $P < 0.05$, Fig. 4B). Ag-specific total Ig (Fig. 5B) and IgG1 (Table 1) levels were siginificantly reduced in CD86−/− mice, while IgG2a remained unchanged (Table 1).

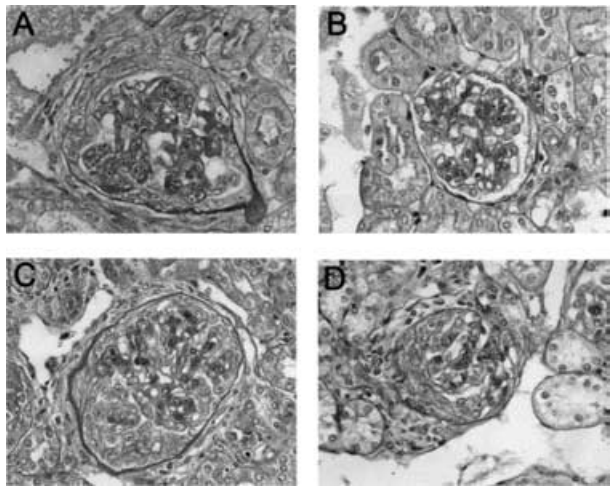

Fig. 2. Representative photomicrographs of glomerular crescent formation in wild-type C57BL/6 controls, CD80−/−, CD86−/−, and CD80/86−/− mice developing anti-GBM GN. Crescents were assessed by PAS and hematoxylin staining of 3-μm thick paraffin sections. Control mice (*A*, × 400) developed proliferative and crescentic GN, which was markedly reduced in CD80−/− mice (*B*, × 400), significantly enhanced in CD86−/− mice (*C*, × 400), and unaffected in CD80/86−/− mice (*D*, × 400).

Crescentic GN develops in the absence of CD80 and CD86 costimulation

CD80/86−/− mice developed GN comparable to C57BL/6 controls, as assessed by proteinuria (4.8 ± 1.0 vs. 6.2 ± 1.4 mg/24hours), crescents (Figs. 1C and 2D), and glomerular macrophages (Fig. 1A) and CD4+ T cells (Fig. 1B). They also had a trend toward increased serum creatinine (21.7 ± 0.9 vs. 31.8 ± 4.2 μmol/L). Apoptosis (3.2 ± 0.1 vs. 1.1 ± 0.1%, $P < 0.001$, Fig. 3B) and proliferation (22.7 ± 2.2 vs. 12.2 ± 0.8%, $P < 0.001$, Fig. 3D) of splenic CD4+ cells was reduced in CD80/86−/− mice compared to C57BL/6 controls. CD80/86−/− mice had decreased splenocyte production of IFN-γ (177 ± 86 vs. 16 ± 7 pg/mL, $P = 0.068$, Fig. 4A) and IL-4 (12.4 ± 5.0 vs. 1.2 ± 0.6 pg/mL, $P < 0.05$, Fig. 4B) compared to C57BL/6 controls. Ag-specific total Ig (Fig. 5C) and IgG1 (Table 1) were significantly attenuated in CD80/86−/− mice, while IgG2a was unaffected (Table 1).

Blockade of OX40-L differentially affects disease outcome in wild-type and CD80/86−/− mice

Inhibition of OX40-L in wt mice enhanced the development of GN compared to wt controls receiving rat IgG,

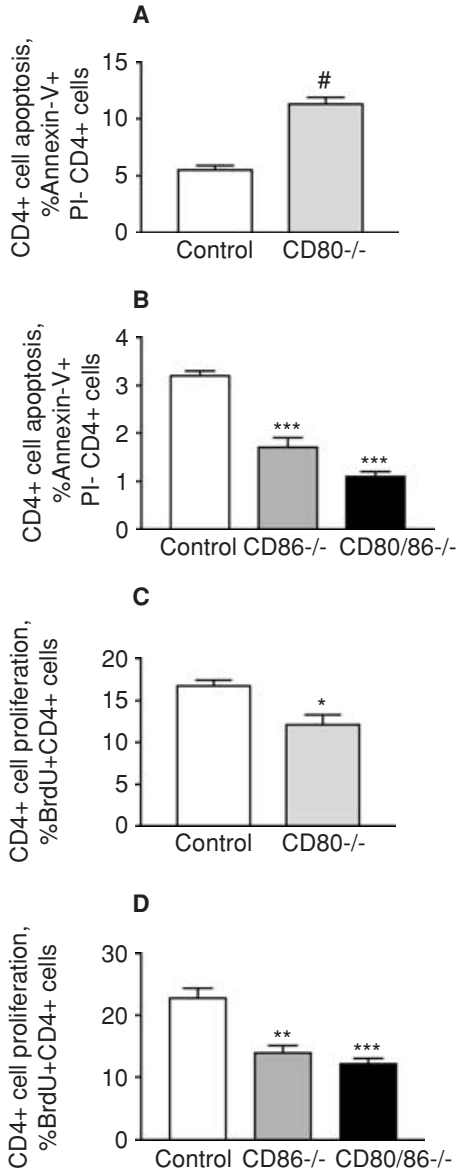

Fig. 3. Flow cytometric analysis of apoptosis and in vivo proliferation of spleen CD4+ T cells from C57BL/6 controls, CD80−/−, CD86−/−, and CD80/86−/− mice developing GN. Apoptosis was measured by Annexin-V staining and proliferation by BrdU labeling. Compared to controls, apoptosis was enhanced in CD80−/− (*A*) and decreased in CD86−/− and CD80/86−/− mice (*B*). Proliferation was decreased in all three groups of knockouts (B and *D*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, # $P < 0.0001$.

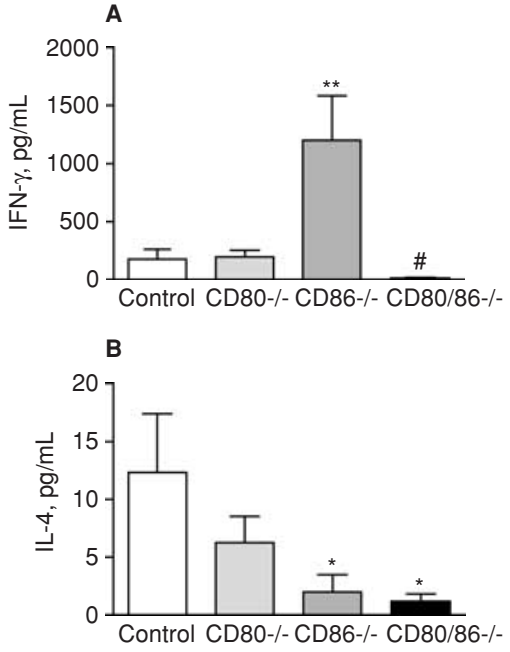

Fig. 4. Production of IFN-γ and IL-4 by splenocytes cultured in the presence of sheep globulin for 72 hours from control mice with anti-GBM GN and from mice deficient in CD80, CD86, and both CD80 and CD86. Levels of IFN-γ (*A*) and IL-4 (*B*) were not significantly different in CD80−/− mice compared to controls. CD86−/− mice had significantly increased production of IFN-γ and reduced IL-4, while the production of both cytokines was markedly attenuated in CD80/86−/− mice compared to controls. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, # $P = 0.06$ vs. control.

as indicated by glomerular infiltration of macrophages (0.9 ± 0.1 vs. 1.1 ± 0.04 c/gcs, $P < 0.01$, Fig. 6A) and CD4+ T cells (0.41 ± 0.03 vs. 0.53 ± 0.03 c/gcs, $P < 0.05$, Fig. 6B), as well as crescent formation (21.7 ± 0.6 vs. $33.7 \pm 1.6\%$, $P < 0.001$, Figs. 6C, 7A and B) and a trend toward increased proteinuria (4.4 ± 0.5 vs. 6.0 ± 0.4 mg/24 h). Wt mice receiving anti-OX40-L Ab treatment had increased production of IFN-γ by Ag-stimulated splenocytes (76 ± 2 vs. 252 ± 18 pg/mL, $P < 0.001$, Fig. 8A), while IL-4 levels (Fig. 8B) were not affected. Apoptosis and proliferation of splenic CD4+ cells were not significantly affected by anti-OX40-L Ab treatment compared wt controls receiving rat IgG (Fig. 9). Ag-specific total serum Ig (Fig. 10) and IgG1 (Table 2) levels were significantly enhanced in wt mice treated with anti-OX40-L Ab compared to wt controls receiving rat IgG, while IgG2a (Table 2) was unaffected.

In contrast, blockade of OX40-L in CD80/86−/− mice attenuated proteinuria (5.1 ± 0.7 vs. 2.7 ± 0.6 mg/24 hours, $P < 0.05$) and crescent formation (23.2 ± 1.2 vs. $14.6 \pm 1.1\%$, $P < 0.001$, Figs. 6C, 7C, and D), as well as glomerular accumulation of macrophages (0.9 ± 0.05 vs. 0.7 ± 0.06c/gcs, $P < 0.05$, Fig. 6A) and CD4+ T cells (0.41 ± 0.03 vs. 0.13 ± 0.02 c/gcs, $P < 0.001$, Fig. 6B) compared to CD80/86−/− controls treated with rat IgG. Interestingly, blockade of OX40-L in CD80/86−/− mice resulted in enhanced apoptosis (1.1 ± 0.1 vs. $5.6 \pm 0.5\%$, $P < 0.001$, Fig. 9A) and attenuated proliferation (12.2 ± 0.8 vs. $6.9 \pm 0.6\%$, Fig. 9B) of splenic CD4+ cells compared to CD80/86−/− controls receiving rat IgG. CD80/86−/− mice treated with anti-OX40-L Ab had significantly diminished production of IFN-γ and IL-4 by Ag-challenged splenocytes compared to wt mice receiving rat IgG or anti-OX40-L Ab; however, their IFN-γ and IL-4 levels

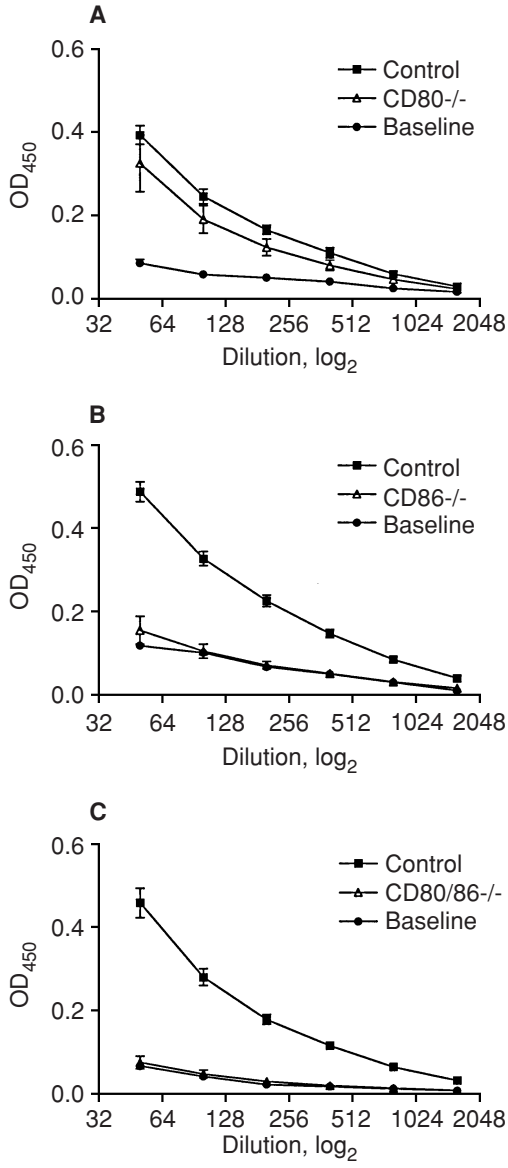

Fig. 5. Levels of circulating mouse anti-sheep globulin Ig in C57BL/6 controls with GN and in CD80−/−, CD86−/−, and CD80/86−/− mice. Serum Ig levels were measured by ELISA and tested at 6 dilutions (1:50 to 1:1600). Baseline represents Ag-specific Ig levels in normal C57BL/6 mice without GN. Ig levels were not significantly different in CD80−/− compared to controls (*A*), but were significantly reduced in CD86−/− ($P < 0.0001$ at all dilutions, *B*) and CD80/86−/− mice ($P < 0.0001$ at all dilutions, *C*) compared to controls.

were not different to CD80/86−/− controls receiving rat IgG (Fig. 8). Similarly, serum levels of Ag-specific total Ig (Fig. 10), as well as IgG1 and IgG2a (Table 2), were markedly attenuated in CD80/86−/− mice treated with anti-OX40-L Ab compared to wt mice receiving rat IgG or anti-OX40-L Ab; however, they were not different when compared to CD80/86−/− controls receiving rat IgG.

Table 1. Circulating levels of antigen-specific IgG1 and IgG2a in CD80−/−, CD86−/−, and CD80/86−/− mice

	IgG1	IgG2a
Normal C57BL/6	0.006 ± 0.006	0.078 ± 0.010
C57BL/6 controls with GN	1.395 ± 0.191	0.252 ± 0.137
CD80−/−	1.042 ± 0.242	0.218 ± 0.116
CD86−/−	0.176 ± 0.084[a]	0.131 ± 0.043
CD80/86−/−	0.138 ± 0.036[a]	0.286 ± 0.187

Serum levels of IgG1 and IgG2a specific to sheep globulin were measured by ELISA at a dilution of 1:50 in normal C57BL/6 mice without GN, C57BL/6 controls with GN, and in CD80−/−, CD86−/−, and CD80/86−/− mice. Results are expressed as the mean OD_{450nm} ± SEM.

[a] $P < 0.0001$ vs. C57BL/6 controls with GN.

Expression of CD80, CD86, and OX40-L

The expression of CD80 was not affected on splenocytes from C57BL/6 compared to CD86−/− mice ($2.3 \pm 0.09 \times 10^7$ vs. $2.2 \pm 0.04 \times 10^7$ cells). Similarly, CD86 expression was not different between C57BL/6 and CD80−/− mice ($1.0 \pm 0.08 \times 10^8$ vs. $1.0 \pm 0.06 \times 10^8$ cells). OX40-L expression was reduced in CD80/86−/− compared to C57BL/6 mice ($9.4 \pm 0.27 \times 10^7$ vs. $6.9 \pm 0.15 \times 10^7$ cells, $P < 0.0001$).

DISCUSSION

The aim of the present study was to investigate the mechanisms by which CD80 and CD86 regulate the development of Th1-driven, Ab-independent crescentic anti-GBM globulin GN, and to determine the role of an alternative costimulatory, OX40/OX40-L pathway in crescentic GN in the absence of CD80 and CD86. Here we show that CD80 and CD86 differentially regulate the development of crescentic anti-GBM GN. Disease was attenuated in the absence of CD80 and exacerbated in the absence of CD86, suggesting that CD80 is pathogenic and CD86 protective in crescentic GN. Similarly, blocking CD80 prevented, whereas inhibition of CD86 enhanced, EAE [11]. The results obtained in CD80−/− and CD86−/− mice cannot be attributed to changes in CD86 and CD80 expression in these mice, respectively, as the expression of CD80 was not different between C57BL/6- and CD86-deficient mice, and CD86 expression was similar between C57BL/6 and CD80−/− mice.

Alterations in the Th1/Th2 balance can affect the development of crescentic GN [30]. CD80 and CD86 have been shown to affect Th1/Th2 differentiation, preferentially regulating Th1 and Th2 development, respectively [11, 31]. Attenuated development of GN in CD80−/− mice cannot be attributed to changes in Th1/Th2 regulation, as indicated by splenocyte IFN-γ and IL-4 production, and circulating IgG1 and IgG2a levels. However, reduced disease correlated with increased CD4+ T cell apoptosis and decreased proliferation, suggesting that CD80 plays a pathogenic role in crescentic GN by enhancing T-cell survival and expansion. Previous studies have shown that

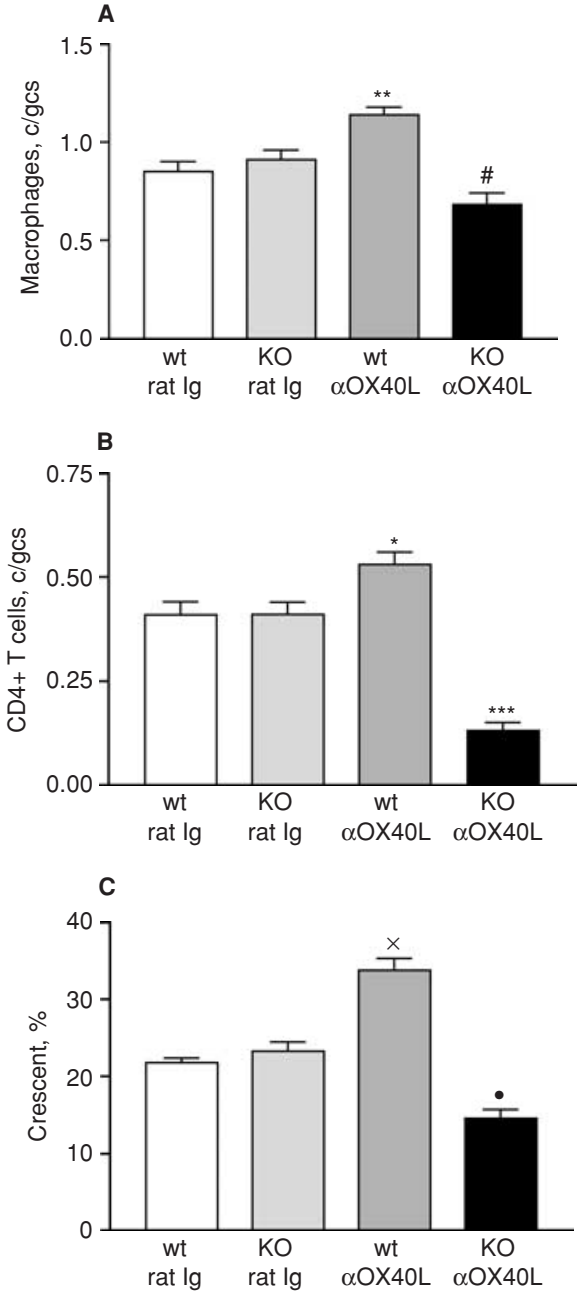

Fig. 6. Glomerular macrophages, CD4+ T cells, and crescents in wild-type (wt) and CD80/86−/− mice receiving rat IgG or anti-OX40-L Ab. Macrophages (*A*), CD4+ T cells (*B*), and crescents (*C*) were significantly enhanced in wt mice treated with anti-OX40-L Ab compared to wt controls receiving rat IgG, and markedly reduced in CD80/86−/− mice treated with anti-OX40-L Ab compared to CD80/86−/− controls receiving rat IgG. c/gcs, cells per glomerular cross section; wt rat Ig, wt controls receiving rat IgG; KO rat Ig, CD80/86−/− controls receiving rat IgG; wt αOX40L, wt mice receiving anti-OX40-L mAb; KO αOX40L, CD80/86−/− mice receiving anti-OX40L mAb. $^{*}P < 0.05$ vs. wt rat Ig, $^{**}P < 0.01$ vs. wt rat Ig, $^{***}P < 0.001$ vs. wt rat Ig, KO rat Ig, and wt αOX40L, $^{\#}P < 0.05$ vs. KO rat Ig and $P < 0.001$ vs. wt αOX40L, $^{\times}P < 0.001$ vs. wt rat Ig, $^{\bullet}P < 0.01$ vs. wt rat Ig and $P < 0.001$ vs. wt αOX40L and vs. KO rat Ig.

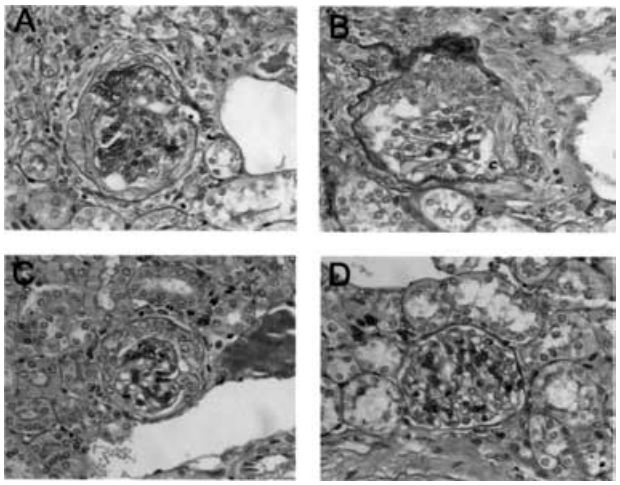

Fig. 7. Representative photomicrographs of glomerular crescent formation in wild-type (wt) and CD80/86−/− mice with anti-GBM GN receiving either control Ab (rat IgG) or anti-OX40L mAb. Crescents were assessed by PAS and hematoxylin staining of 3-μm thick paraffin sections. (*A*) Wt controls receiving rat IgG (× 400); (*B*) wt mice receiving anti-OX40L Ab (× 400); (*C*) CD80/86−/− controls receiving rat IgG (× 400); (*D*) CD80/86−/− mice receiving anti-OX40L Ab (× 400). Anti-OX40L mAb treatment enhanced crescent formation in wt mice compared to wt controls receiving rat IgG. CD80/86−/− mice treated with anti-OX40L Ab attenuated crescent formation compared to wt and CD80/86−/− mice receiving rat IgG.

B7 molecules can promote T-cell survival and expansion and, as such, augment disease [8, 32–35].

In contrast, the absence of CD86 caused a shift toward Th1 responses, as indicated by reduced IL-4 and enhanced IFN-γ production, and decreased IgG1 levels, suggesting that CD86 plays a protective role in crescentic GN by promoting Th2 and consequently attenuating Th1 responses. Similarly, anti-CD86 mAb treatment increased the severity of EAE by enhancing Th1 differentiation [11]. These observations are consistent with the majority of evidence demonstrating that CD86 engagement is more critical for Th2 than Th1 responses [31, 36]. In contrast, when CD86 was blocked with mAb in anti-GBM GN, a change in Th1/Th2 balance was not observed [26], probably due to a transient and incomplete inhibition of CD86. Proliferation and apoptosis of splenic CD4+ T cells were both reduced to a similar extent in CD86−/− mice, suggesting that enhanced disease in these mice cannot be attributed to an increase in overall T-cell viability. Decreased apoptosis in CD86−/− mice is surprising because previous evidence has demonstrated that B7 molecules promote T-cell survival [8, 32–35]; however, as will be discussed later, this may be due to the compensatory role of the OX40/OX40-L costimulatory pathway.

Mice lacking both CD80 and CD86 had disease similar to controls, suggesting that anti-GBM GN can fully develop in the absence of CD80/86 costimulation. Other immune-mediated diseases, such as experimental autoimmune myocarditis, can also develop in the absence of

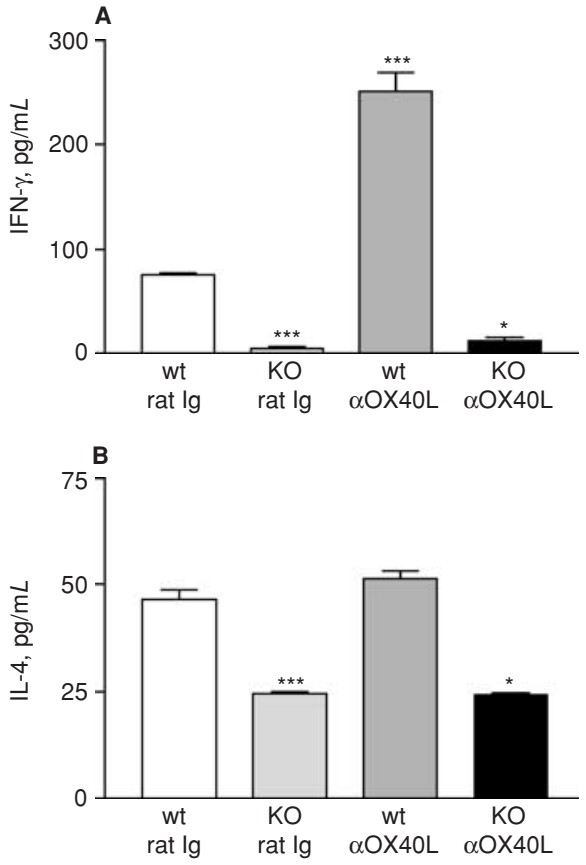

Fig. 8. Production of IFN-γ and IL-4 by splenocytes cultured in the presence of sheep globulin for 72 hours from wild-type (wt) and CD80/86−/− mice receiving rat IgG or anti-OX40-L Ab. Anti-OX40-L Ab treatment significantly increased IFN-γ (*A*) and did not affect IL-4 (*B*) levels in wt mice. CD80/86−/− mice receiving anti-OX40-L Ab had significantly reduced IFN-γ and IL-4 compared to wt mice receiving anti-OX40-L Ab, but similar levels of both cytokines when compared to CD80/86−/− controls receiving rat IgG. wt rat Ig, wild-type controls receiving rat IgG; KO rat Ig, CD80/86−/− controls receiving rat IgG; wt αOX40L, wild-type mice receiving anti-OX40-L mAb; KO αOX40L, CD80/86−/− mice receiving anti-OX40L mAb. $^{*}P < 0.001$ vs. wt rat Ig and vs. wt αOX40L, $^{***}P < 0.001$ vs. wt rat Ig.

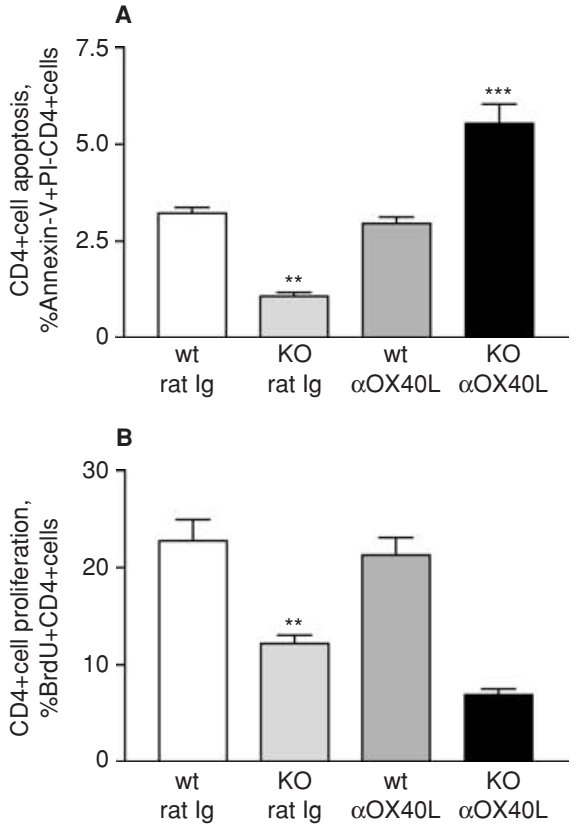

Fig. 9. Flow cytometric analysis of apoptosis and proliferation of splenic CD4+ T cells from wild-type (wt) and CD80/86−/− mice receiving anti-OX40-L Ab or rat IgG. Apoptosis was measured by Annexin-V staining and proliferation by BrdU labeling. Blockade of OX40-L in wt mice did not affect apoptosis (*A*) or proliferation (*B*) of CD4+ cells compared to wt controls receiving rat IgG. Inhibition of OX40-L in CD80/86−/− mice enhanced apoptosis (A) and decreased proliferation (B) of CD4+ cells compared to CD80/86−/− controls receiving rat IgG. wt rat Ig, wild-type controls receiving rat IgG; KO rat Ig, CD80/86−/− controls receiving rat IgG; wt αOX40L, wild-type mice receiving anti-OX40-L mAb; KO αOX40L, CD80/86−/− mice receiving anti-OX40L mAb. $^{**}P < 0.001$ vs. wt rat IgG, $^{***}P < 0.001$ vs. wt rat IgG, KO rat IgG, and KO αOX40L.

CD28-mediated costimulation [37]. Conversely, in other models of GN, EAG, and lupus nephritis, disease was ameliorated in the absence of CD80 and CD86 [18–20, 38]. It is possible that autoreactive T cells in EAG and lupus nephritis are potentially more dependent on CD80/86 costimulation due to their weaker affinity for Ag because CD28 costimulation is more critical under conditions of suboptimal T-cell activation [39]. Attenuated EAG and lupus nephritis were also associated with decreased intrarenal Ab deposits, which are pathogenic in these models of GN [40, 41], but are not required for the development of anti-GBM GN [1].

CD80/86−/− mice developed GN comparable to controls despite decreased production of IFN-γ and IL-4. However, proliferation (46% decrease) and, to a larger extent, apoptosis (67% decrease) of splenic CD4+ T cells were significantly reduced in CD80/86-deficient mice, suggesting that the overall increased survival of CD4+ cells in CD80/86−/− mice may explain why these mice had disease similar to controls. Reduced apoptosis in CD80/86−/− mice is unexpected; however, further data from the current study demonstrate that this is due to compensation by OX40-L–mediated costimulation.

To investigate whether the OX40/OX40-L pathway is an alternative costimulatory pathway in the absence of CD80 and CD86 during the development of anti-GBM GN, OX40-L was blocked in wt and CD80/86−/− mice developing anti-GBM GN. Inhibition of OX40-L in wt mice exacerbated, whereas OX40-L blockade in CD80/86−/− mice attenuated GN, suggesting that OX40-L differentially regulates the development of GN depending on the presence or absence of CD80 and CD86.

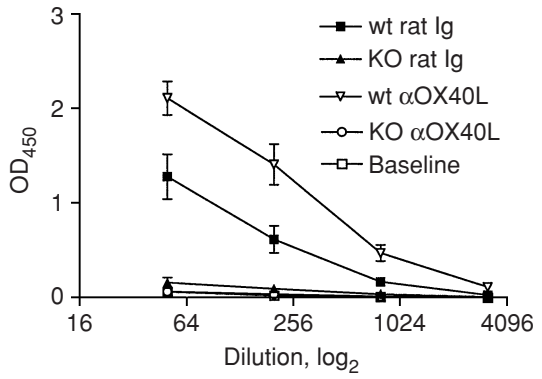

Fig. 10. Antigen-specific circulating Ig in wild-type (wt) and CD80/86−/− mice receiving either control rat IgG or anti-OX40-L Ab. Serum Ig levels were measured by ELISA and tested at 4 dilutions (1:50 to 1:1600). Baseline represents Ig levels in nonimmunized C57BL/6 controls without GN. Inhibition of OX40-L significantly increased Ig levels in wt mice ($P < 0.01$ vs. wt rat Ig at all dilutions). Ig levels in CD80/86−/− mice were markedly attenuated by anti-OX40-L Ab treatment ($P < 0.001$ vs. wt rat Ig and vs. wt αOX40L at all dilutions); however, they were not different when compared to CD80/86−/− controls receiving rat IgG. wt rat Ig, wt controls receiving rat IgG; KO rat Ig, CD80/86−/− controls receiving rat IgG; wt αOX40L, wt mice receiving anti-OX40-L mAb; KO αOX40L, CD80/86−/− mice receiving anti-OX40-L mAb.

Table 2. Circulating levels of antigen-specific IgG1 and IgG2a in wild-type (wt) and CD80/86−/− mice receiving either rat IgG or anti-OX40-L mAb

	IgG1	IgG2a
Wt + rat IgG[a]	2.012 ± 0.124	0.966 ± 0.450
CD80/86−/− + rat IgG[b]	0.173 ± 0.129[e]	0.038 ± 0.009[h]
Wt + anti-OX40-L Ab[c]	2.476 ± 0.033[f]	0.984 ± 0.455[h]
CD80/86−/− + anti-OX40-L Ab[d]	0.021 ± 0.011[g]	0.006 ± 0.003[h]

Serum levels of IgG1 and IgG2a specific to sheep globulin were measured by ELISA at a dilution of 1:50 in wt and CD80/86−/− mice receiving either rat IgG or anti-OX40-L mAb. Results are expressed as the mean OD_{450nm} ± SEM.

[a]Wt + rat IgG: wild-type controls with GN receiving rat IgG.

[b]CD80/86−/− + rat IgG: CD80/86−/− controls with GN receiving rat IgG.

[c]Wt + anti-OX40-L Ab: wild-type mice with GN receiving anti-OX40-L mAb.

[d]CD80/86−/− + anti-OX40-L Ab: CD80/86−/− mice with GN receiving anti-OX40-L mAb.

[e]$P < 0.001$ vs. Wt + rat IgG; [f] $P < 0.05$ vs. Wt + rat IgG; [g] $P < 0.001$ vs. Wt + rat IgG and vs. wt + anti-OX40-L Ab; [h] $P > 0.05$ vs. all the groups.

In the presence of CD80 and CD86, OX40-L attenuates GN, whereas in their absence, it enhances disease, suggesting that OX40/OX40-L signaling compensates for the absence of CD80 and CD86. Similarly, blockade of OX40-L reduced disease in EAE-susceptible CD28−/− mice, whereas it had no effect on disease development in wt controls [14]. Other costimulatory pathways, including CD40/CD40-L and ICOS/ICOS-L, are not excluded from playing a role in CD28-independent immune responses inducing crescentic GN because they can also provide an alternative activation signal to T cells in the absence of B7-mediated costimulation [42–44].

Inhibition of OX40-L in wt mice resulted in elevated IFN-γ and unaffected IL-4 production by splenocytes, indicating that, in the presence of CD80 and CD86, OX40-L plays a protective role in GN by down-regulating the Th1 response. In contrast, levels IFN-γ and IL-4 in CD80/86−/− mice treated with anti-OX40-L mAb were comparable to those in CD80/86−/− controls receiving rat IgG, suggesting that reduced disease in CD80/86−/− mice treated with anti-OX40L Ab cannot be explained by changes in cytokine production. Proliferation and apoptosis of CD4+ T cells were not affected by OX40-L blockade in wt mice, suggesting that these mechanisms are not responsible for exacerbated disease in these animals. Quite interestingly, however, inhibition of OX40-L in CD80/86−/− mice enhanced CD4+ T-cell apoptosis and decreased proliferation, implying that in the absence of CD80 and CD86, OX40-L plays a pathogenic role in GN by decreasing apoptosis and increasing proliferation of T cells. These results indicate that reduced apoptosis in CD80/86−/− mice compared to wt controls is a consequence of compensation by OX40-L signaling, and is probably due to the absence of CD86 because mice deficient in CD86 and not CD80 also had decreased apoptosis and enhanced disease. OX40-L expression was significantly reduced in the absence of CD80 and CD86, suggesting that the observed effects of OX40-L blockade in CD80/86−/− mice are real and would be even greater if OX40-L expression was comparable between CD80/86−/− and C57BL/6 mice.

A recent study by Takeda et al has demonstrated that OX40 signaling is not only critical for activation of naïve nonregulatory T cells, but is also important for the suppressive function of CD4+CD25+ regulatory T cells, or Tregs [45]. Furthermore, it was shown that CD28 signaling is required for OX40 to overcome Treg-mediated suppression. These observations are, therefore, potentially relevant to the results of the current study. However, we found that, in GN, OX40-L is protective in the presence and pathogenic in the absence of CD80/86, suggesting that effect of OX40-L inhibition on Tregs is probably not responsible for the differential role of OX40-L in wt compared to CD80/86−/− mice.

Circulating Ig levels were not affected in CD80−/− mice, but were reduced to baseline levels in the absence of CD86 alone and both CD80 and CD86. These results are consistent with previous studies demonstrating that CD86 has a more dominant role in Ig production [23, 46], and suggest that CD80 is not required, whereas CD86 is essential for T-cell–dependent humoral immunity. Altered Ig levels, however, are not likely to explain disease outcome in the current study because autologous Ab is not required for the development of crescentic anti-GBM GN [1]. In fact, the present study further confirms that anti-GBM GN is Ab-independent because CD86−/− mice, which had very little Ab, developed exacerbated disease. In addition, this study shows that in the presence of CD86-mediated signaling, OX40/OX40-L interaction down-regulates Ab production. Collectively, these results

suggest that CD86 and OX40-L differentially regulate T-cell–dependent humoral immunity in crescentic GN.

CONCLUSION

The present study shows that CD80 and CD86 differentially regulate the development of Th1-mediated, Ab-independent crescentic anti-GBM GN and do so by different mechanisms. CD80 has a pathogenic role in this disease by decreasing apoptosis and increasing proliferation of nephritogenic CD4+ T cells, whereas CD86 plays a protective role by enhancing Th2 and attenuating Th1 responses. Furthermore, the current study demonstrates that OX40-L differentially regulates the development of GN depending on the presence or absence of CD80 and CD86 and does so by different mechanisms. In the presence of CD80 and CD86, OX40-L has a protective role by attenuating Th1 responses, whereas in the absence of these molecules, it plays a pathogenic role by enhancing survival and expansion of nephritogenic T cells. Therefore, the OX40/OX40-L pathway is an alternative costimulatory pathway for mediating immune responses that induce crescentic GN in the absence of CD80 and CD86.

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